



TITLE:

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Disruption of *lig4* improves gene targeting efficiency in the oleaginous fungus *Mortierella alpina* 1S-4

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Abbreviations: HR, homologous recombination; NHEJ, none homologousend joining; DSBs,
DNA double-strand breaks; OA, oleic acid; LA, linoleic acid; GLA, γ -linolenic acid;
DGLA, dihomo- γ -linolenic acid; ARA, arachidonic acid.

Highlights

- We identified a *lig4* gene encoding DNA ligase 4 in *Mortierella alpina* 1S-4
- We disrupted the *lig4* gene in *M. alpina* 1S-4 by homologous recombination
- We improved gene targeting efficiency dramatically in *M. alpina* 1S-4 by
disruption of the *lig4* gene

Abstract

The oil-producing zygomycete *Mortierella alpina* 1S-4 is known to accumulate beneficial polyunsaturated fatty acids. We identified the *lig4* gene that encodes for a DNA ligase 4 homolog, which functions to repair double strand breaks by non-homologous end joining. We disrupted the *lig4* gene to improve the gene targeting efficiency in *M. alpina*. The *M. alpina* 1S-4 Δ *lig4* strains showed no defect in vegetative growth, formation of spores, and fatty acid production, but exhibited high sensitivity to methyl methanesulfonate, an agent that causes DNA double-strand breaks. Importantly, gene replacement of *ura5* marker by *CBXB* marker occurred in 67% of Δ *lig4* strains and the gene targeting efficiency was 21-fold greater than that observed in disruption of the *lig4* gene in the *M. alpina* 1S-4 host strain. Further metabolic engineering of the Δ *lig4* strains is expected to result in strains that produce higher levels of rare and beneficial polyunsaturated fatty acids and contribute to basic research on the zygomycete.

Keywords

Mortierella alpina; DNA ligase 4 (*lig4*); homologous recombination; gene targeting

1. Introduction

The integration of exogenous DNA into chromosomes occurs through two pathways that repair DNA double-strand breaks (DSBs), homologous recombination (HR) and non-homologous end joining (NHEJ) (Kanaar et al., 1998). These pathways are independent of one another and often function competitively (Van Dyck et al., 1999).

In HR, exogenous DNA is integrated into the chromosome by making use of homologous regions as templates for precise insertion. This process requires the Rad family of proteins, (Rad54, Rad51, Rad52), Mre11, and Xrs2 (Kooistra et al., 2004; Krappmann, 2007). HR is largely conserved among species and has been ubiquitously found from bacteria and yeast to humans (Krogh and Symington, 2004; Shibata et al., 2001).

NHEJ, on the other hand, involves direct ligation of the exogenous DNA strand ends into the DSBs and has no requirement for sequence homology. In general, NHEJ requires Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), DNA ligase 4 (Lig4), and X-ray repair cross-complementing protein 4 (Xrcc4) (Critchlow and Jackson, 1998; Daley et al., 2005). NHEJ is also largely conserved among species. It was reported that a Ku8-dependent and Ku80-independent NHEJ manner was found in *Neurospora*, whereas the Lig4 was a key enzyme related with all

NHEJ pathways (Ishibashi et al., 2006). Most organisms, including mammals, plants, insects, and filamentous fungi, predominantly utilize the NHEJ pathway for DSB repair. Recently, it was reported that disruption of the *ku70*, *ku80* or *lig4* genes leads to an increase in the frequency of HR in various filamentous fungi (Ishidoh et al., 2014; Maruyama and Kitamoto, 2008; Ninomiya et al., 2004). In particular, disruption of the *lig4* gene in *Neurospora crassa* and *Aspergillus oryzae* increased targeting efficiency to 100% (Ishibashi et al., 2006; Mizutani et al., 2008).

The oil-producing filamentous fungus, *Mortierella alpina* 1S-4, is capable of producing large amounts of carbon 20 (C20) polyunsaturated fatty acids (PUFAs), such as arachidonic acid (20:4 ω 6, ARA) and the eicosapentaenoic acid (20:5 ω 3, EPA) in triacylglycerols (Sakuradani et al., 2009). This fungus has been used as a model microorganism for the biosynthesis and accumulation of lipid molecules, including PUFAs (Kawashima et al., 1995; Kikukawa et al., 2013; Sakuradani, 2010; Sakuradani et al., 2013). We previously reported techniques for gene manipulation in this fungus (Ando et al., 2009a; Ando et al., 2009b; Takeno et al., 2004a, 2004b; Takeno et al., 2005b) to further understand the selective production of various PUFAs in *M. alpina* 1S-4. Genes were manipulated by means of chemical mutation with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine as well as molecular breeding through

overexpressing or silencing genes by RNA interference (Sakuradani, 2010; Sakuradani et al., 2013; Takeno et al., 2005a). However, chemical mutation and molecular breeding have difficulty in isolating mutations producing various PUFAs and in controlling the biosynthetic pathways of PUFAs in transformants, respectively. Furthermore, traditional mutagenesis yields undesirable mutations in the genome, and plasmid vectors are randomly integrated, which often unnecessarily affect growth rate and/or fatty acid production of this fungus.

Previously, we disrupted the *ku80* gene by means of biolistic particle bombardment (Ando et al., 2009a) and evaluated the improvement in gene targeting efficiency in the $\Delta ku80$ strain. However, significant improvement was not detected (Kikukawa et al., 2015). In this study, we sought to develop a more efficient gene targeting system in *M. alpina* 1S-4 by disrupting the *lig4* gene through double crossing-over HR by the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method. The ATMT method introduces a single-copy of the fragment between the left and right borders of gene disruption vectors into the genome. It was previously reported that the ratio of integration by HR was increased by ATMT method (Gouka et al., 1999; Kito et al., 2008; Michielse et al., 2005a; Michielse et al., 2005b). In the ATMT of *M. alpina* 1S-4, integration at multiple sites in the genome occurs at low frequencies in *M. alpina* 1S-4

(Ando et al., 2009b).

2. Materials and methods

2.1 Enzymes and chemicals

Restriction enzymes and other DNA-modifying enzymes were obtained from Takara Bio Inc. (Shiga, Japan). All other chemicals were of the highest purity commercially available.

2.2 Strains, media, and growth conditions

M. alpina 1S-4 is deposited in the culture collection of the Graduate School of Agriculture of Kyoto University (Sakuradani, 2010) and the uracil auxotrophic (*ura5*[−]) strain with a mutation in the *ura5* gene selected from *M. alpina* 1S-4 was used as the host strain in this study (Takeno et al., 2004b). Czapek-Dox agar medium containing 0.05 mg/ml uracil was used for sporulation of *M. alpina* 1S-4 *ura5*[−] strain (Takeno et al., 2004b). Synthetic complete (SC) medium was used as a uracil-free synthetic medium for cultivation of the transformants derived from *M. alpina* 1S-4 *ura5*[−] strain at 28°C (Takeno et al., 2004b). GY medium (2% glucose and 1% yeast extract) was used for fatty acid composition analysis and extraction of genomic DNA. Uracil-free SC agar plates containing 0.03% Nile Blue A (Sigma-Aldrich, St. Louis, MO) and GY agar

plates containing 10 µg/ml carboxin and 0.03% Nile Blue A were used for selection of transformants with the carboxin resistance *CBXB* gene cassette (Ando et al., 2009a). *Escherichia coli* DH5α was used for DNA manipulation and grown on LB agar plates containing 50 µg/ml kanamycin. *Agrobacterium tumefaciens* C58C1 was used for the transformation of the *M. alpina* 1S-4 *ura5⁻* strain. LB-Mg agar medium, minimal medium (MM), and induction medium (IM) were used for transformation, cultivation, and infection of *A. tumefaciens*, respectively (Ando et al., 2009b). All solid media were made by addition of 2.0% agar.

2.3 Genomic DNA preparation

M. alpina 1S-4 was cultivated in 100 ml of GY liquid medium for 5 days at 28°C with shaking at 100 rpm. Preparation of genomic DNA was performed as previously described (Okuda et al., 2014b).

2.4 Cloning and identification of the *lig4* gene from *M. alpina* 1S-4

Two highly degenerate primers, Lig4 F1 and Lig4 R1, were synthesized for cloning the *lig4* gene (Table S1). The primers were designed based on the conserved amino acid sequences of Lig4 homologs from *Rhizopus delemar* (accession no. **EIE79010**), *Canis*

lupus (accession no. **XP_542663**), *Laccaria bicolor* (accession no. **XP_001874645**),
Pan troglodytes (accession no. **XP_509726**), *Schizosaccharomyces pombe* (accession no.
NP_587888). The sequences of the sense and antisense primers correspond to the
regions that encode LIVGGY and TLRFPF peptides, respectively. PCR amplification
was carried out in a total volume of 50 μ l containing 1 μ g of genomic DNA, 0.25 μ l of
Takara EX Taq polymerase (Takara Bio Inc.), 5 μ l of 10 \times *EX Taq* buffer, 200 μ M of
each dNTP, and 5 pM of each primer, and conditions were as follows: 35 cycles of 1
min at 94°C, 1 min at 60°C, and 2 min at 72°C, followed by a final extension period of 5
min at 72°C. The resulting 571 bp fragment was cloned into the pT7Blue T-Vector
(Novagen, Madison, WI, USA) and the sequence was verified using the
Beckman-Coulter CEQ8000 system (Beckman-Coulter, Fullerton, CA, USA). RNA was
isolated using Isogen (Nippon Gene, Tokyo, Japan) and cDNA was synthesized using
the PrimeScript™ High Fidelity RT-PCR Kit (Takara Bio Inc.), according to the
manufacturers' protocols.

To isolate the entire *lig4* genomic DNA from *M. alpina* 1S-4, inverse PCR was
performed using Lig4 IPCR F and Lig4 IPCR R primers (Table S1). The
*Bam*HI-digested genomic fragment was self-ligated and used as a template for PCR
amplification. PCR amplification was carried out in a total volume of 50 μ l containing

500 ng of the template, 0.25 μ l of *Takara EX Taq* polymerase (Takara Bio Inc.), 5 μ l of $10 \times EX Taq$ buffer, 200 μ M of each dNTP, and 5 pM of each primer, and conditions were as follows: initial denaturation of 1 min at 94°C, 35 cycles of 40 sec at 94°C, 40 sec at 57°C, and 3 min at 72°C, and an extension period of 10 min at 72°C. The amplified 3.0 kb fragment was cloned into the pT7Blue T-Vector and the sequence was confirmed using the Beckman-Coulter CEQ8000 system.

2.5 Construction of *lig4* gene targeting plasmid vectors

A T-DNA binary vector for *lig4* gene targeting was constructed using the *M. alpina* 1S-4 transformation vector pBIG35Zh (Ando et al., 2009b; Okuda et al., 2014a) or pBIG3CBZ. pBIG3CBZ was generated by excising the carboxin resistance *CBXB* gene cassette (Ando et al., 2009a) from the *M. alpina* 1S-4 transformation vector pSDZNCBXB using the *Eco*RI and *Xba*I restriction sites. The resulting fragment was ligated into the pBIG35Zh vector using the same restriction sites, and the resulting plasmid was designated pBIG3CBZ. The 5'-*lig4* fragment (1.6 kb) and 3'-*lig4* fragment (1.8 kb) were amplified from the *M. alpina* 1S-4 genomic DNA template using Lig4 F1 *Apa*I and Lig4 R1 *Apa*I, and Lig4 F2 *Xba*I and Lig4 R2 *Nhe*I (Table S1), respectively. pBIG35Z Δ L4 and pBIG3CBZ Δ L4 were generated by ligating the 3'-*lig4* fragment

(*Xba*I and *Nhe*I restriction sites) and the 5'-*lig4* fragment (*Apa*I restriction site) into pBIG35Zh and pBIG3CBZ, respectively (Fig. 1). pBIG35Z Δ L4 is used for disruption of the *lig4* gene in the host strain, and pBIG3CBZ Δ L4 is used for estimating the targeting efficiency in a Δ *lig4* strain. The replacement of the *ura5* marker with the *CBXB* marker in the *lig4* locus was confirmed by PCR using the extracted genomic DNA as a template and Lig4 up F and CBXB stop R primers (Table S1). The sequences between left border (LB) and right border (RB) integrate into the genomic DNA of *M. alpina* 1S-4 upon transformation.

2.6 Transformation of *M. alpina* 1S-4

Spores of the *M. alpina* 1S-4 uracil auxotrophic strain was freshly prepared from cultures growing on Czapek-Dox agar medium supplemented with 0.05 mg/ml uracil, and the spore suspension was filtered through Miracloth (Calbiochem) (Takeno et al., 2004b).

Transformation of *M. alpina* 1S-4 was performed using the ATMT method as previously described (Ando et al., 2009b) with slight modification. After co-cultivation of *A. tumefaciens* cells and *M. alpina* 1S-4 spores, the membranes were transferred to uracil-free SC agar plates containing 0.03% Nile Blue A or GY agar plates containing

10 µg/ml carboxin. After incubation for 4 days at 28°C, aerial hyphae of fungal colonies were transferred to fresh uracil-free SC agar plates or GY agar plates containing 100 µg/ml carboxin.

2.7 Selection of *lig4* gene-disrupted strains by PCR

M. alpina 1S-4 transformed with pBIG35ZΔL4 were cultured on fresh uracil-free SC agar medium at least three times. Candidates containing *lig4* gene disruption were then identified by first amplifying the *lig4* genomic gene locus using Lig4 up F and Lig4 down R primers (Table S1) and genomic DNA prepared from the transformants. Then, the resulting fragment was used as a template for another round of PCR amplification using three primer sets, Lig4 F1 *Apa*I and Lig4 R1 *Apa*I, Lig4 in F and Lig4 in R, and Lig4 F1 *Apa*I and *ura5* 400 R (Table S1 and Fig. S1).

2.8 Southern blot analysis

Southern blot hybridization was carried out as previously described (Ando et al., 2009b). Genomic DNA (10 µg) was digested with *Sac*I and *Xho*I, size-fractionated by electrophoresis (1% agarose), and transferred to Amersham Hybond-N⁺ membrane (GE Healthcare Ltd., Buckinghamshire, UK). Probes used for hybridization correspond to a

0.8 kb region of the 5'-fragment of *lig4* gene and a 0.6 kb region of the *ura5* gene, and were generated using Lig4 F2 and Lig4 R1 *Apa*I, and *ura5* start F and *ura5* stop R primer sets (Table S1), respectively. Southern hybridization was performed with Amersham Alkphos Direct Labeling Reagents (GE Healthcare) and Amersham CDP-Star Detection Reagent (GE Healthcare), and chemiluminescence was detected by ImageQuant LAS 4000 mini system (GE Healthcare).

2.9 Mutagen sensitivity of Δ lig4::*ura5* strain

Sensitivity to chemical mutagens was evaluated by the spot test (Kato et al., 2004; Mizutani et al., 2008). Methyl methanesulfonate (MMS), at 0, 0.01, 0.02, 0.03, 0.04, and 0.005% [w/v], was added to SC agar medium supplemented with 0.05 mg/ml uracil. Spores were spotted and grown on the agar plates for 8 days.

2.10 Fatty acid analysis

Analysis of the fatty acid production and compositions of the transformants was performed as previously described (Kikukawa et al., 2013) with slight modification. The mycelia of the Δ lig4 strain and its host strain were inoculated into 10 ml of GY liquid medium and cultivated for 7 days at 28°C with reciprocal shaking at 300 rpm. The

fungal strains were harvested by filtration and dried for 3 h at 120°C. The dried cells were directly transmethylated with 10% methanolic HCl for 2 h at 55°C. The resulting fatty acid methyl esters (FAMES) were extracted using *n*-hexane, concentrated, and analyzed by GC-2010 Plus gas chromatography (GC; Shimadzu, Kyoto, Japan) equipped with a TC-70 capillary column (GL Science Inc., Tokyo, Japan). Fatty acid quantification was calculated using tricosanoic acid as an internal standard. All experiments were performed three times.

2.11 Nucleotide sequence accession number

The *M. alpina* 1S-4 *lig4* gene is registered in the DNA Data Bank of Japan (DDBJ) database under the accession number **LC009448**.

3. Results

3.1 Identification and phylogenetic analysis of the *M. alpina* 1S-4 *lig4* homolog

The open reading frame (ORF) of the *M. alpina* *lig4* gene consists of 3,309 bp of DNA spanning ten exons (1-103, 217-691, 791-901, 1,009-1,212, 1,312-1,354, 1,449-1,659, 1,776-1,881, 1,974-2,079, 2,178-2,872, and 2,964-3,309). The predicted *M. alpina* Lig4 protein consists of 799 amino acids and shares low sequence identity with

Lig4 from other ascomycetes (*Aspergillus nidulans*, 32%; *Aspergillus oryzae*, 31%; *Aspergillus fumigatus*, 29%), basidiomycetes (*Cryptococcus neoformans*, 24%; *Coprinopsis cinerea*, 29%), mammals (*Mus musculus*, 31%; *Rattus norvegicus*, 30%; *Homo sapiens*, 31%), and higher plants (*Arabidopsis thaliana*, 26%; *Oryza sativa*, 33%).

The phylogenetic tree of various homologous Lig4 proteins from these organisms indicated that Lig4 homologs are clustered in each organism group, such as fungi, mammals, and plants, whereas the Lig4 protein (MaLig4) from *M. alpina* 1S-4 was remotely related to those from other organisms (Fig. 2).

3.2 Identification and characterization of Δ lig4 mutants

To generate a Δ lig4 strain (Δ lig4::ura5) of *M. alpina* 1S-4, spores were transformed with pBIG35Z Δ L4 (Fig.1) using the ATMT method (Fig. 3A). Then, the *lig4* disruptants were selected by PCR analysis. The primers Lig4 F1 *Apa*I and Lig4 R1 *Apa*I resulting in the 1.6 kb fragment of 5'-*lig4* upstream region were used for control. When disruption of the *lig4* gene locus occurs, the 0.2 kb fragment from the intact *lig4* gene is no longer amplified by the Lig4 in F and Lig4 in R primers. Instead, a 2.5 kb fragment is amplified in reactions using Lig4 F1 *Apa*I and ura5 400 R primers, indicating that the fragment is successfully integrated into the *lig4* gene locus (Fig.

S1A). A schematic showing the location of the primers used to identify gene disruption and a representative transformant showing the results of the PCR assay are shown in Fig. S1A and S1B. We screened 93 transformants by PCR and found that 3 transformants showed *lig4* gene disruption.

We then investigated whether a single homologous integration of the disruption construct into the *lig4* gene correctly occurred by Southern blot analysis. Locations of the probes used for the Southern blot analysis are depicted in Fig. 3A. After digestion of genomic DNA with *Xho*I and detection with probe A, we observed the expected 2.9 kb fragment in the host *lig4* gene whereas the gene disrupted mutant had a 1.0 kb fragment (Fig. 3B). We also observed the expected 1.9 kb fragment in *lig4* disrupted genomic DNA digested with *Xho*I, and the 10.7 kb fragment in *Sac*I digested genomic DNA when probe B was used (Fig. 3B). These results suggested that gene replacement with *ura5* marker had successfully occurred at the *lig4* gene locus in the host strain of *M. alpina* 1S-4. The targeting efficiency of the *lig4* gene in the uracil auxotrophic strain was calculated to be 3.2% (Table 1).

3.3 Estimate of gene targeting efficiency in the Δ *lig4* mutant

To evaluate the improvement of the gene targeting efficiency in the *Δlig4::ura5*

strain, we performed an assay to measure the replacement of *ura5* marker with the *CBXB* marker in the *Δlig4* locus (Fig. 4A). The pBIG3CBZΔL4 vector was introduced into spores of the *Δlig4::ura5* strain grown on GY medium containing 100 μg/ml carboxin by the ATMT method. Integration of the *CBXB* gene was measured by colony PCR where successful replacement resulted in the amplification of a 3.5 kb fragment (Fig. 4A and 4B). Of the three transformants analyzed, two showed the expected replacement in the *Δlig4* locus. We also confirmed replacement by sequencing using the Beckman-Coulter CEQ8000 system (data not shown). We calculated the targeting efficiency to be 66.7%, which is 20.7-fold greater than the isolation of the *Δlig4* strain from *M. alpina* 1S-4 host strain (Table 1).

3.4 Mutagen sensitivity, growth, and fatty acid productivity of the *Δlig4::ura5* strain

The *Δlig4::ura5* strain showed sufficient sporulation, and the spores germinated on MMS-free SC agar medium. However, compared to the host strain, the *Δlig4::ura5* strain grew at a significantly decreased rate when grown on SC agar plates containing MMS (Fig. 5). The *Δlig4::ura5* strain was not capable of growing on the plates containing MMS concentrations higher than 0.03%, whereas the host strain was able to grow at 0.03% MMS. Furthermore, we compared the production of fatty acids and

growth rates of the *Δlig4::ura5* strain to those of host strain. The *Δlig4::ura5* and host strains showed similar growth rates and slightly higher fatty acid production in GY liquid medium (Table 2), and both also formed white aerial hyphae (under condition of 0% MMS in Fig. 5). The *Δlig4::ura5* strain showed similar PUFA composition mainly consisting of linoleic acid (LA), γ -linoleic acid (GLA), dihomo- γ -linolenic acid (DGLA), and ARA, to that of the host strain (Table 2).

4. Discussion

We cloned and identified the *lig4* gene encoding DNA ligase 4 (Lig4), a protein involved in NHEJ of all species, in *M. alpina* 1S-4. Homologous Lig4 proteins identified in other organisms were classified by kingdom and showed that the Lig4 homolog (MaLig4) from *M. alpina* 1S-4 shares low identities (about 30%) with those of other organisms (Fig. 2).

We then developed a method to disrupt the *lig4* gene (the $\Delta lig4::ura5$ strain) in *M. alpina* 1S-4 through HR with pBIG35Z Δ L4 vector. By using the ATMT method, we obtained $\Delta lig4::ura5$ strains with 3.2% efficiency which is a slightly higher than the biolistic particle bombardment method used for *ku80* gene disruption (Kikukawa et al., 2015). Additionally, the targeting efficiency of the replacement of *ura5* marker by the *CBXB* marker in the $\Delta lig4$ locus in the $\Delta lig4::ura5$ strain was calculated as 66.7%, which is 20.7-fold higher than disruption of the *lig4* gene with pBIG35Z Δ L4 in *M. alpina* 1S-4 host strain (Table 1). Compared with that of the $\Delta ku80$ strain, the targeting efficiency was drastically improved in $\Delta lig4$ strain (Kikukawa et al., 2015). The disruption of *ku80* was performed through single crossing-over HR, resulting in incomplete *ku80* disruptant. It was suggested that the incomplete Ku80 protein formed a Ku-protein complex with the Ku70 protein and reduced targeting efficiency. One

explanation for this improvement of targeting efficiency is that disruption of *lig4* gene through double crossing-over HR led to complete inactivation of Lig4. However, unlike *N. crassa*, *A. oryzae*, and *A. luchuensis*, the targeting efficiency did not reach 100% in the $\Delta lig4::ura5$ strain (Ishibashi et al., 2006; Mizutani et al., 2008; Takahashi et al., 2011). It is unclear why the gene targeting efficiency in several organisms, including this fungus, did not improve to 100% (Kito et al., 2008; Maruyama and Kitamoto, 2008; Tashiro et al., 2013). Depending on the target gene locus, the targeting efficiency is quite different because of the mosaic nature of active and inactive domains in eukaryotic chromosomes (Wolffe and Guschin, 2000). Therefore, we need to evaluate the gene targeting efficiency by disruption of various genes related to fatty acid synthesis in order to collect more statistical data.

Lig4 protein is involved in NHEJ in phylogenetically diverse organisms (Hopfner et al., 2002; Lisby and Rothstein, 2004) and in telomere maintenance in some organisms (Hande, 2004). The *lig4* disruption in some organisms led to sensitivity to MMS (Ishibashi et al., 2006; Ninomiya et al., 2004). The $\Delta lig4::ura5$ strain exhibited high sensitivity to MMS (Fig. 5), and the sensitivity was similar to that of *N. crassa*, *A. oryzae*, and *A. kawachii* (Ishibashi et al., 2006; Mizutani et al., 2008; Tashiro et al., 2013). The high sensitivity of the $\Delta lig4::ura5$ strain to MMS suggests repression of the

NHEJ pathway in this strain.

This study is the first report to describe the identification and characterization of the *lig4* gene from *Mucoromycotina*. The $\Delta lig4::ura5$ strain showed no defect in vegetative growth, formation of spores, and fatty acid productivity (Table 2) making it suitable for subsequent molecular breeding. Additionally, we demonstrated improvement in gene targeting efficiency in the $\Delta lig4$ strain, which is expected to facilitate metabolic engineering for production of beneficial PUFAs and reverse genetic studies for basic research in *M. alpina* 1S-4.

5. Conclusion

We successfully improved the efficiency of gene targeting in *M. alpina* 1S-4 by disrupting the *lig4* gene. These novel $\Delta lig4$ mutants display similar growth rate, sporulation, and production of PUFA to that of the host strain. This study is the first report to describe the identification and characterization of *lig4* gene from Mucoromycotina. Our efficient gene targeting system may be used to generate various gene disrupted strains to produce beneficial PUFAs and may also reveal new mechanisms of lipid biosynthesis and lipid accumulation.

Conflict of Interest

The authors declare no conflict of interests.

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Figure legends

Fig. 1. The pBIG35ZΔL4 or pBIG3CBZΔL4 vector used for *lig4* gene-disruption. LB, left border; RB, right border; *His550p*, *M. alpina* 1S-4 histone H4.1 promoter short fragment; *ura5*, orotate phosphoribosyltransferase gene of *M. alpina* 1S-4; *CBXB*, the mutated *sdhB* gene for carboxin resistance; *SdhBt*, *M. alpina* 1S-4 *sdhB* translation terminator; *NPTIII*, neomycin phosphotransferase III gene; *TrfA*, *trfA* locus, which produces two proteins that promote replication of the plasmid; *ColEI ori*, ColEI origin of replication; *oriV*, pRK2 origin of replication.

Fig. 2. The phylogenetic analysis of Lig4 homologs. The tree was created by using the neighbor-joining (NJ) method with 10,000 bootstrap replicates by the sequence analysis software GENETYX ver. 11.0 (Genetyx corp., Tokyo, Japan). *Aspergillus nidulans* (AnLig4), **Q5BH83**; *Aspergillus oryzae* (AoLig4), **BAE62914**; *Aspergillus fumigatus* (AfLig4), **EAL91408**; *Cryptococcus neoformans* (CnLig4), **AAW46139**; *Coprinopsis cinerea* (CcLig4), **Q7Z7W5**; *Mus musculus* (MmLig4), **NP_795927**; *Rattus norvegicus* (RnLig4), **NP_001099565**; *Homo sapiens* (HsLig4), **NP_001091738**; *Arabidopsis thaliana* (AtLig4), **NP_568851**; *Oryza sativa* (OsLig4), **Q7X7E9**; *Mortierella alpina* 1S-4 (MaLig4), **LC009448**.

Fig. 3. Schematic for disruption of the *lig4* gene showing the probe locations for Southern blot analysis and confirmation of Δ*lig4* strains by Southern blot. (A) The figure illustrates homologous integration of the pBIG35ZΔL4 vector into the *lig4* genomic gene locus in *M. alpina* 1S-4. Gray short bar indicates the position hybridized

by probe. *Dotted lines* indicate the position and base length of hybridization signals. (B) Southern hybridization analysis of the *Alig4* gene strains. *XhoI*- or *SacI*-digested genomic DNA was hybridized with the probes shown in A. The asterisks demonstrate signals of an endogenous *ura5* gene. Lane 1, host (*ura5*⁻) strain; Lane 2, *Alig4* strain.

Fig. 4. Evaluation of gene targeting efficiency. (A) The figure illustrates homologous integration of the pBIG3CBZΔL4 vector into the *Alig4* genomic gene locus in the *Alig4::ura5* strain. *Black arrows*, a and b indicate the position of oligonucleotide primers used for PCR. (B) Confirmation of the replacement of the *ura5* marker to the *CBXB* marker by PCR. PCR amplification was carried out with Lig4 up F (a) and *CBXB* stop R (b) primers and results in a 3.5 kb product. Lane 1, the *Alig4::ura5* strain used as a host strain; Lane 2, the strain having the *Alig4* locus replaced with the *CBXB* marker from the *Alig4::ura5* strain; Lane M, marker.

Fig. 5. Sensitivity of *M. alpina* 1S-4 host strain and *Alig4* strain to methyl methanesulfonate (MMS). Spores were cultivated on SC agar plates with or without MMS for 8 days and colony diameter was measured.

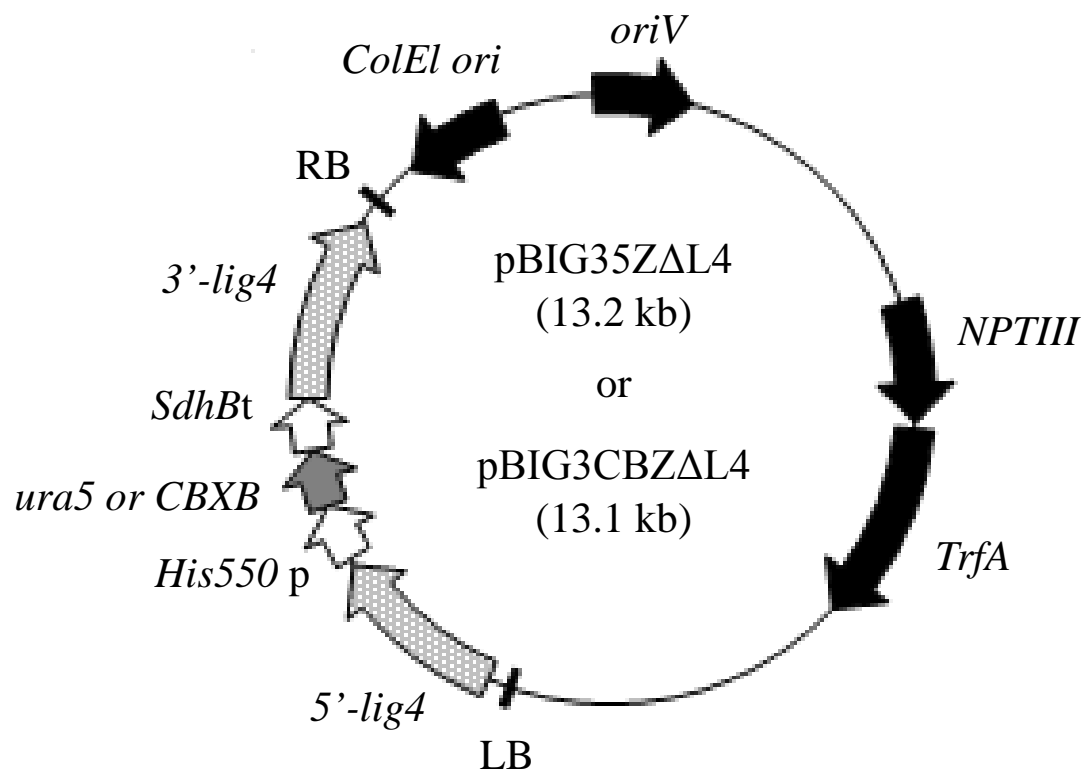


Fig. 1 Kikukawa et al.

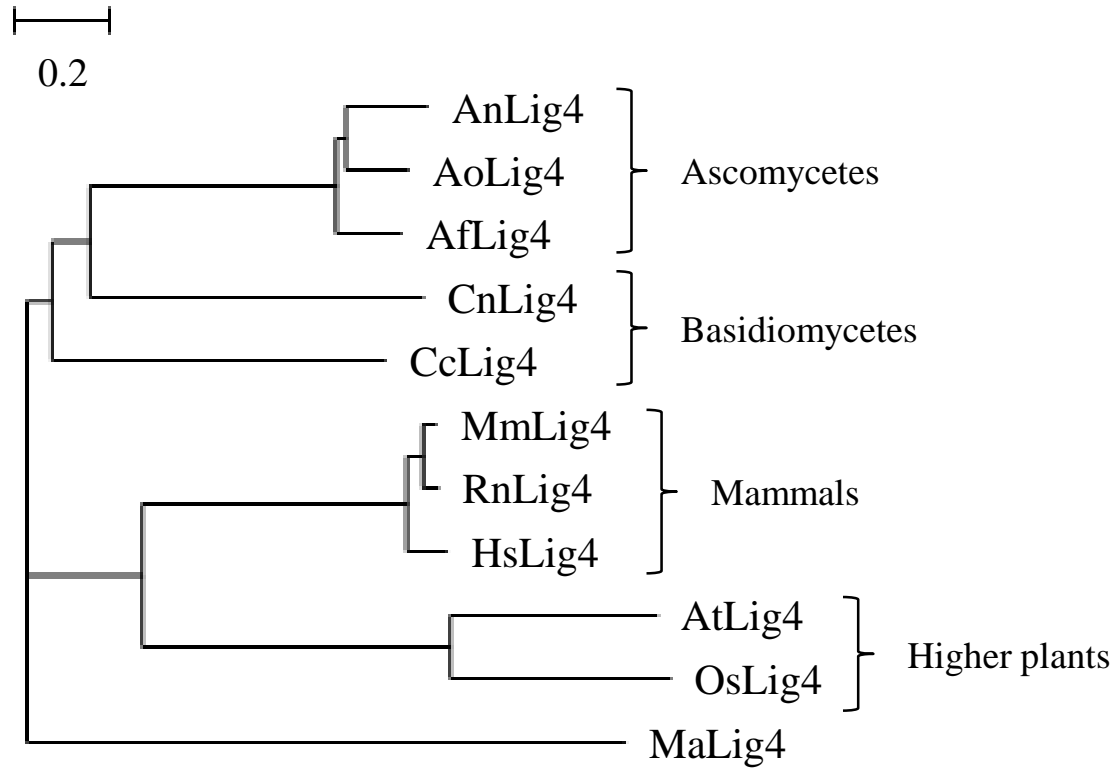


Fig. 2 Kikukawa et al.

A

lig4-targeting DNA
with the *ura5* marker

Host strain
genomic DNA

Δ *lig4* locus with
the *ura5* marker

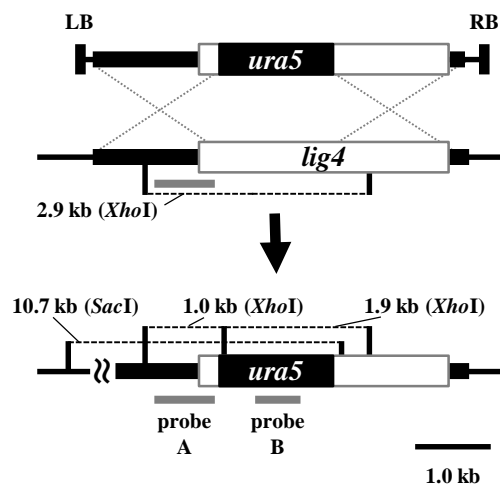


Fig. 3A Kikukawa et al.

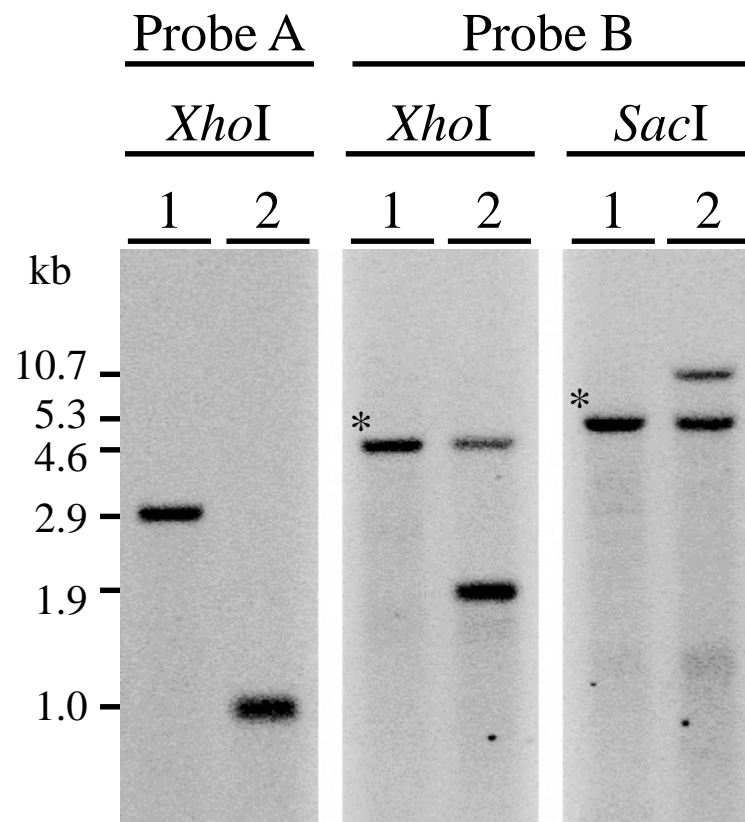


Fig. 3B Kikukawa et al.

A

lig4-targeting DNA
with the *ura5* marker

Host strain
genomic DNA

Alig4 locus with
the *CBXB* marker

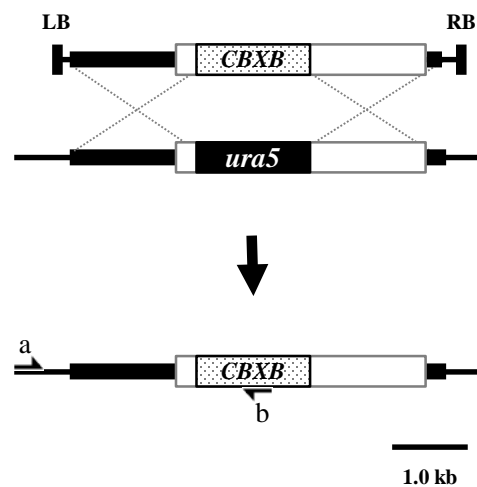


Fig. 4A Kikukawa et al.

B

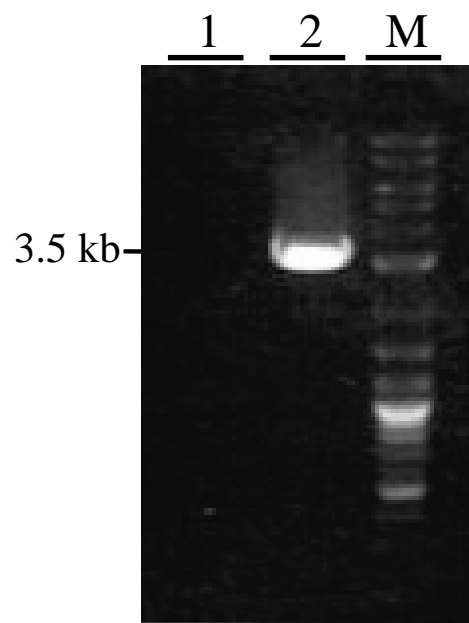


Fig. 4B Kikukawa et al.

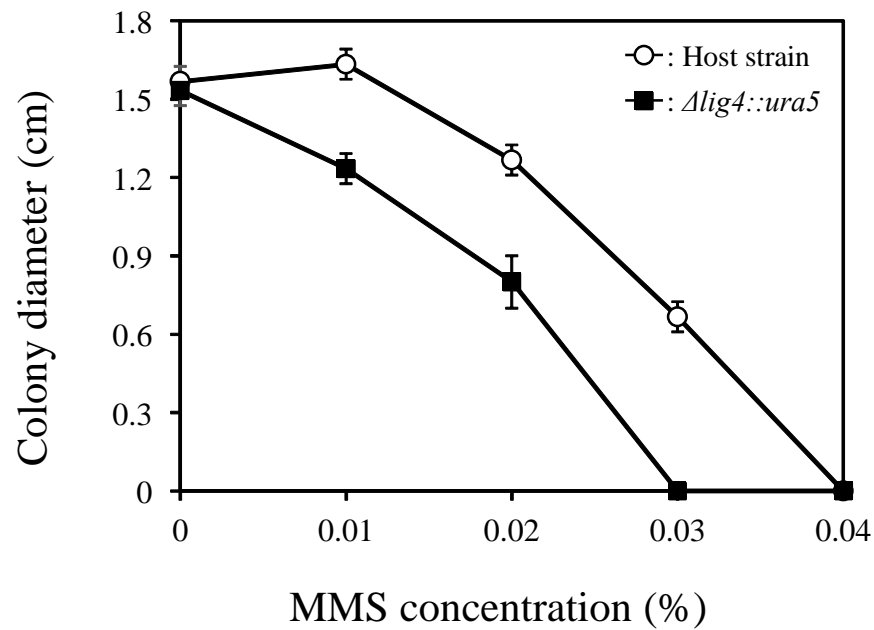


Fig. 5 Kikukawa et al.

1 **Table S1** Primers used in this study

| 2 | Primer | Sequence (5' to 3') |
|----|----------------------|---|
| 3 | Lig4 F1 | YTIATYGTBGTBTA |
| 4 | Lig4 R1 | AAYYTIMGITTYCCIMG |
| 5 | Lig4 IPCR F | CCAGAGAGGTATGCTGCACCTATTTTACACCAACCAATCC |
| 6 | Lig4 IPCR R | GCCAAACATCTCGAACGTAATTCCTGATCCCCTTTGCAGAAGGTC |
| 7 | Lig4 F1 <i>Apa</i> I | GCGGGCCCTGACCTAACAGCCCCGACAATAC |
| 8 | Lig4 R1 <i>Apa</i> I | GCGGGCCCAATATCATGTATCACACAATGTCAG |
| 9 | Lig4 F2 <i>Xba</i> I | GCTCTAGAAGACAGCCGTCCTCATACG |
| 10 | Lig4 R2 <i>Nhe</i> I | GCGCTAGCAACTACTTCGACACCGCAAC |
| 11 | Lig4 up F | GTGTGCCATCAACACGTTGTCTGC |
| 12 | Lig4 down R | CCAAGAACGACATGTCAATGTACG |
| 13 | Lig4 in F | TCCACAGATCTTCCCAAAGG |
| 14 | Lig4 in R | CGATCAGCACGTCCAAACTA |
| 15 | ura5 400 R | CATGGTCCTTCTTTTCCTTGC |
| 16 | Lig4 F2 | GCTGTTACGGCTTACCATC |
| 17 | ura5 start F | GGATTACCAGCGCGAGTTC |
| 18 | ura5 stop R | TTAAACACCGTACTTCTCGCGG |
| 19 | CBXB stop R | GATTACTCCAAAGCCATGGTC |

- 1 “The underlined sequences show synthesized restriction enzyme sites.

lig4-targeting DNA
with the *ura5* marker

Host strain
genomic DNA

Δ *lig4* locus with
the *ura5* marker

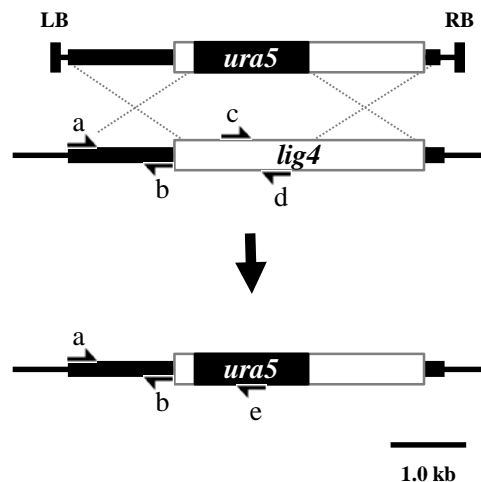


Fig. S1. Schematic for disruption of the *lig4* gene showing PCR primer location and confirmation of Δ *lig4* strains by PCR. (A) The figure illustrates integration of the pBIG35Z Δ L4 vector into the *lig4* genomic gene locus in *M. alpina* 1S-4. Black arrows, a ~ e, indicate the position of oligonucleotide primers used for PCR: a, Lig4 F1 ApaI; b, Lig4 R1 ApaI; c, Lig4 in F; d, Lig4 in R; e, *ura5* 400 R. (B) Selection of Δ *lig4* strains by PCR. Lane 1, primers a and b; Lane 2, primers c and d; Lane 3, primers a and e.

